REMARKS

Claims 1-21 are pending in the application. Claims 1-8 are withdrawn from consideration as being drawn to a non-elected invention, and Claims 9-21 are rejected. By the present amendment, Claims 1-8 and 21 are canceled without prejudice or disclaimer, and Claims 9, 11, 12, 13, and 15-20 are amended. By the present amendment, new Claims 22-40 are added. As the specification, including the figures, support the amendments and new claims, the amendments and new claims add no new matter.

In view of the above-described amendments and following remarks, reconsideration of claims 9-20, and consideration of new claims 22-40 are respectfully requested.

§112 Rejections

Claims 9-16 are rejected under 35 USC §112, first paragraph, "because the specification while being enabling for activated form of vitamin D binding protein (ADBP) and fADBP (SEQ ID NO:1) does not reasonably provide enablement for one or more DBP peptides and combinations thereof." (See last paragraph on page 2 of the Office Action.)

Claim 9 as amended recites a method of increasing bone density in a subject in need of the same by administering ADBP. As the Patent Office has stated, such method is enabled. Claims 10 and 17-20 depend from claim 9, and are also enabled.

Claim 11 is amended to recite a method of increasing bone density in a subject in need of the same by administering a peptide comprising the first 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, or 14 amino acids of SEQ ID NO. 1. The present application provides sufficient guidance for one of ordinary skill in the art to make such peptides. Moreover, the present application shows that administration of such peptides increases the total bone density, trabecular bone density, or cortical bone density in newborn or adult rats. (See Figures 2, 8, 9, 10 ,11, and 12 of the present application.) That the claimed peptides can be used for this increasing bone density in a mammalian subject is further confirmed by the studies described in the articles authored by one or more of the named inventors, and attached hereto as Appendices A, B, C, D, E, F, and G. Claims 12-16 depend from claim 11, and are also enabled.

Accordingly, Applicants submit that the rejection of claims 9-16, as not being enabled should be withdrawn.

Claim 21 is rejected under 35 USC § 112, second paragraph as being indefinite. Although applicants do not agree with the Patent Office's assessment regarding the definiteness of Claim 21, Claim 21 has been canceled in order to expedite prosecution of the present application. Accordingly, the rejection is moot.

§ 103 Rejections

Claims 9-16 are rejected as being unpatentable over Yamamoto (USPN 6,410,269) (hereinafter "Yamamoto").

Claim 9 has been amended to recite a method of increasing bone density in a subject in need of the same by administering ADBP to the subject, and claim 11 has been amended to recite a method of increasing bone density in a subject in need of the same by administering a peptide that comprises the first 3-8 or 10-14 amino acids of SEQ ID NO. 1 to the subject. Yamamoto neither teaches nor suggests such a method. Yamamoto recites that the recombinant protein and specific peptide taught therein "are to be used for therapy of cancer, HIV-infection and osteopetrosis". Thus, the only bone disorder mentioned in Yamamoto is osteopetrosis, a condition which, according to Yamamoto, is "characterized by an excess accumulation of bone throughout the skeleton..." (See column 4, lines 41-42 of Yamamoto. Emphasis added.). Because they have excess bone, patients with osteopetrosis are not in need of a therapy that increases bone density. In addition, osteopetrosis is not a disease or disorder associated with bone loss or increased activity or numbers of osteoclasts. Rather, osteopetrosis is associated with "deficient or dysfunctional osteoclasts". (See column 4, lines 51-53 of Yamamoto.) Thus, Yamamoto would not motivate one of ordinary skill in the art to treat a patient that has systemic or localized bone loss with the recombinant vitamin D binding protein recited in Yamamoto or any fragment thereof. Lacking such motivation, Yamamoto does not render claims 9 or 11, or the claims that depend therefrom obvious. Moreover, Applicants also note that the only peptide disclosed in Yamamoto column 2, lines 4-7 and column 8, lines 46-49 is the 80 amino acid fragment which forms domain III of vitamin D binding protein (See column of Yamamoto)). For this additional reason, Yamamoto does not render the method recited in claims 11 or the

claims that depend therefrom obvious. Accordingly, applicants submit amended claims 9 and 11, and the claims that depend therefrom are patentable over Yamamoto, and that the rejection should be withdrawn.

In view of the above-described amendments and remarks, applicants submit that claims 9-20 and new claims 22-40 are allowable. Prompt notice of such allowance is respectfully requested. If the Examiner feels that further changes to the application are necessary or if he has any questions regarding the amendments or new claims, he is invited to contact the undersigned at the telephone number listed below.

Respectfully submitted,

Date: September 20, 2004

By: Pamela A. Docherty

Pamela A. Docherty, Reg. No. 40,591
(216) 622-8416

APPENDIX A

The Effects of a Group of Novel Anabolic Peptides on Bone Density and Bone Strength in Adult Rats

Gary B. Schneider[1], Michael J. Askew*[2], Kristina J. Grecco*[1], Jason Hsu*[1], Emily Mugler*[2], Donald A. Noe*[2]

[1]Division of Basic Medical Sciences, Northeastern Ohio Universities College of Medicine, Rootstown, OH

[2]Walter A. Hoyt, Jr., Musculoskeletal Research Laboratory, SUMMA Health System, Akron, OH

ABSTRACT

We previously demonstrated that a 14 amino acid fragment from the third domain of the human serum protein, vitamin D binding protein (DBP), when delivered systemically, elicited anabolic effects on bone. Intermittent subcutaneous injections of these 14 a.a. peptides to intact, adult rats resulted in significant increases in total bone density in the long bones with just two weeks of treatment (Schneider et. al., JBMR, 16:S231). In the current study we evaluated peptide fragments, ranging from 3-13 a.a. in length, created by single a.a. deletions from the c-terminal end of our original peptide. Adult, intact female rats were given subcutaneous injections of saline or peptide (0.4 ng/g body wt.) every 48 hrs. for two weeks; two days after the final injections the animals were euthanized, the femurs and tibias analyzed by peripheral quantitative computerized tomography (pQCT) and by three-point bending, biomechanical testing. Specific slices through the metaphysis and mid-shaft of each bone were analyzed from the scans. A number of the peptide fragments elicited responses which included highly significant ($p < 0.001$) increases in total bone density, significant increases in cortical/subcortical bone density, little change in trabecular bone density, decreases in total surface area, and decreases in periosteal and endosteal circumferences. All of the peptide-treated and control bones were subjected to 3-point bending at the mid-shaft, to take advantage of the geometrical data generated by the pQCT analysis, which was subsequently used in the biomechanical analyses. All of the specific peptide fragments which demonstrated highly significant increases in total bone density also demonstrated highly significant increases in bone strength. None of the peptide treatments affected the modulus of the bones as compared to controls. There was a very significant correlation between bone density and strength in the various peptide-treatment groups. The correlation coefficient was 0.60 ($p = 0.002$). In conclusion, those peptide fragments which enhanced bone density also enhanced bone strength, suggesting that the use of these novel peptides results in the generation of superior quality bone.

PEPTIDE CHARACTERISTICS

- Synthetically produced
- Based on human amino acid sequence near the site of glycosylation in the third domain of DBP
- Fragments 3 to 14 amino acids in length
- Novel peptide - no homologies other than DBP

EXPERIMENTAL DESIGN

- S.C. injections of saline or peptide (0.4 ng/g body weight) were given every other day for 2 weeks
- Two days after the final injections, rats were sacrificed
- Femurs and tibiae were collected for bone densitometry
- 3-point bending to determine strength and bending modulus

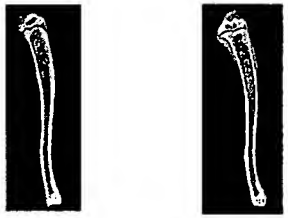

Figure 1 – CT Scans of Bone. These illustrated scans are midline longitudinal slices and represent control and treated adult rats.

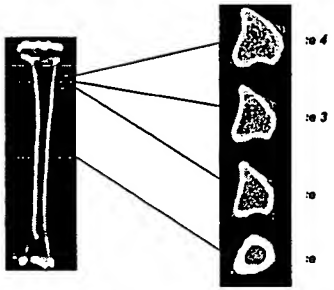

Figure 2 – pQCT Analyses. The image on the left is a longitudinal "scout" view of the tibia of a young adult rat. All analyses of bone density included three slices from the proximal tibial metaphysis and a single mid-shaft slice (Slice 1).

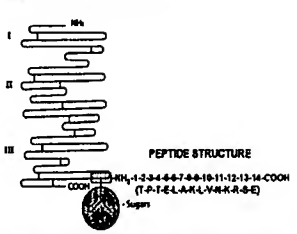

Figure 3 – DBP Protein and Peptide Structure. All peptide fragments were generated by deletions of amino acids from the C-terminal end. The number on the figures represent the number of amino acids in each fragment starting at the N-terminal. All fragments contain the potential glycosylation site on the native protein.

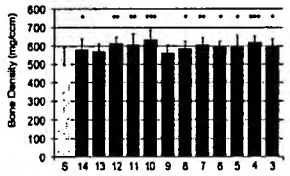

Figure 4 – Total Bone Density. Animals treated with the various peptide fragments demonstrated a range of responses from highly significant increases in bone density to no change. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

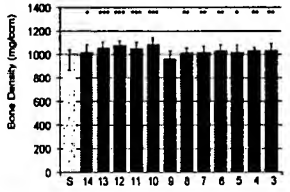

Figure 5 – Cortical/Subcortical Bone Density. Animals treated with the various peptide fragments demonstrated a range of responses from highly significant increases in bone density to no change. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

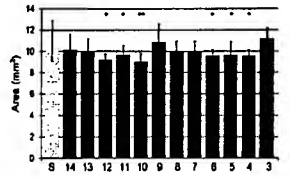

Figure 6 – Total Area. Animals treated with the various peptide fragments demonstrated a range of responses from significant reductions in cross-sectional area of the proximal tibia to no change. * = $p<0.05$, ** = $p<0.01$.

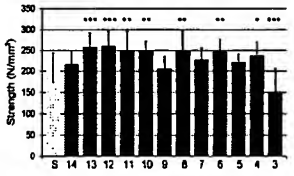

Figure 7 – Bone Strength. Tibial bones were tested to failure in three-point bending. The ultimate strength was calculated from the load deflection data and cross-sectional geometries of the mid-shaft (slice 1). Animals treated with the various peptide fragments demonstrated a range of responses from highly significant increases in strength to reductions in strength. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

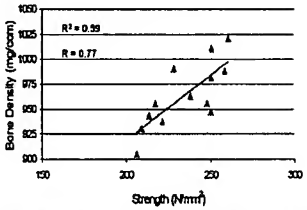

Figure 8 – Bone Density vs. Strength. When bone density was plotted vs. strength there was a highly significant, direct correlation. Those peptide treatments resulting in the most significant increases in bone density in the tibial mid-shaft were also the strongest. $p=0.002$

CONCLUSIONS

- The intermittent injections of a number of the peptide fragments significantly enhance bone density in the long bones of adult rats.
- A major contributor to the increase in total bone density was highly significant increases in cortical/subcortical density.
- Those treatment groups demonstrating the most significant increases in total bone density showed decreases in cross-sectional area of the tibial bones.
- The various peptide fragments had differential effects on bone strength, ranging from highly significant increases in strength to decreases in strength.
- There was a strong correlation between bone density and bone strength among the treatment groups. Those treatments which enhanced bone density also resulted in stronger bones--suggesting better bone quality.

APPENDIX B

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2001 Program & Abstracts

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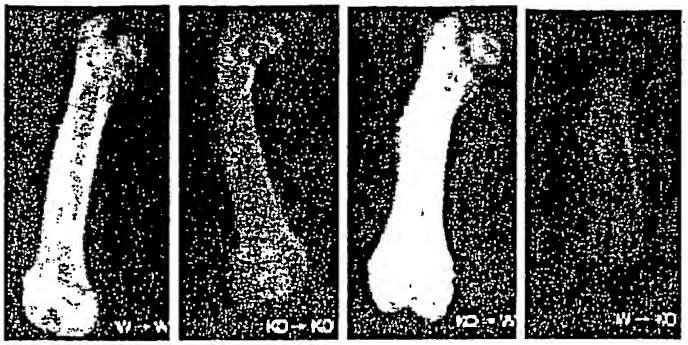

F512

Active Vitamin D Inhibits Osteoclastogenesis by Interfering With AP-1/NF-κB Activity in Osteoclast Precursors. H. Takasu,[1] S. Takeda,*[1] A. Sugita,[1] T. Kake,[1] N. Kubota,*[1] E. Ogata,[2] K. Ikeda,[3] Y. Uchiyama.*[1] [1]Fuji Gotemba Res. Lab., Chugai Pharmaceutical Co., Ltd., Shizuoka, Japan. [2]Cancer Inst. Hosp., Japanese Foundation for Cancer Res., Tokyo, Japan. [3]Dept. of Geriatric Res., Natl. Inst. for Longevity Sci., Aichi, Japan.

We have demonstrated that active vitamin D inhibits bone resorption *in vivo* in estrogen deficient rodent models of high turnover osteoporosis (*JBMR 2000*). This contradicts the prevailing notion that $1,25D_3$ induces RANKL in bone marrow stromal cells, thereby promoting differentiation and activation of osteoclasts *in vitro*. In order to solve this discrepancy and to clarify the mechanism by which active vitamin D inhibits osteoclastic bone resorption, we examined the effects of $1,25D_3$ on osteoclastogenesis induced by M-CSF and RANKL in murine marrow cultures. Bone marrow cells from 6-8-week-old male *ddy* mice were cultured with M-CSF for 3 days, and adherent cells consisted mainly of bone marrow macrophage (BMM) were further cultured with M-CSF and sRANKL for additional 3-5 days. The number of TRAP-positive multinucleated cells (more than 3 nuclei) was counted. Addition of $1,25D_3$ inhibited the formation of osteoclasts dose-dependently, with IC50 being 10^{-9}M and 10^{-10}M $1,25D_3$ inhibiting by 70-80%. The expression of VDR in BMM was confirmed, and $1,25D_3$ had no inhibitory effect in bone marrow cells from VDR knockout mice, pointing to a VDR-mediated process. Addition of $1,25D_3$ during the first 3 days had no effect, while its co-presence with sRANKL during the latter half period fully inhibited osteoclastogenesis, and treatment with $1,25D_3$ did not affect RANK level in BMM, suggesting that $1,25D_3$ acts downstream of RANK activation by RANKL. Phosphorylation of IκB at Ser 32 after treatment with sRANKL was not inhibited by $1,25D_3$. A novel vitamin D analog, DD-28I, that we have identified on the basis of its greater ability to inhibit AP-1/NF-κB-mediated transcription (25-30x of $1,25D_3$) and weaker activity to induce VDRE-dependent transcription (1/10 of $1,25D_3$) inhibited osteoclast formation 10x more potently than $1,25D_3$ (IC50 being 10^{-10}M), raising the possibility that active vitamin D inhibits osteoclastogenesis by interfering with AP-1/NF-κB function in osteoclast precursors through binding to VDR. In conclusion, we think that the major *in vivo* pharmacological action of active vitamin D is not to induce RANKL ("soil") in marrow stromal cells but to inhibit osteoclastic bone resorption by acting on osteoclast precursors ("seeds") and interfering with RANK signaling, and that the latter action provides an attractive target for developing new VDR-based drugs for osteoporosis.

F513

The Anabolic Effect of Vitamin D Binding Protein-Macrophage Activating Factor (DBP-MAF) and a Novel Small Peptide on Bone. G. B. Schneider,[1] K. J. Greeco,*[1] F. F. Safadi,[2] S. N. Popoff.[2] [1]Division of Basic Medical Sciences, Northeastern Ohio Universities College of Medicine, Rootstown, OH, USA. [2]Department of Anatomy and Cell Biology, Temple University School of Medicine, Philadelphia, PA, USA.

Vitamin D binding protein-macrophage activating factor (DBP-MAF) has previously been shown to stimulate bone resorption and correct the skeletal defects associated with osteopenosis in two non-allelic mutations in rats. This same protein and a small fragment of the protein have now been shown to demonstrate an anabolic effect on the skeleton of both newborn and young adult, intact rats. The novel peptide fragment was synthetically produced based on the human amino acid sequence at the site of glycosylation in the third domain of the native protein (DBP). The peptide tested is 14 amino acids in length and demonstrates no homologies other than to that region of DBP. Newborn rats were injected i.p. with saline, peptide (0.4 ng/g body wt.) or DBP-MAF (2 ng/g body wt.) every other day from birth to 14 days of age. On day 16 the rats were euthanized and the long bones collected for bone densitometry by pQCT. Serum was collected for evaluation of osteocalcin levels as an indication of bone formation and urine was analyzed for deoxypyridinoline (Dpd) as a measure of bone resorption. After two weeks of treatment with either the whole protein (DBP-MAF) or the small peptide, bone density was significantly increased in the treated animals compared to the saline controls. Serum osteocalcin levels were significantly enhanced and Dpd levels in the urine were significantly decreased in the protein and peptide treated animals. Young adult female rats were given s.c. injections of saline or peptide (0.4 ng/g body wt. or 5 ng/g body wt.) every other day for two weeks; two days after the final injections, the rats were euthanized and the femurs and tibias collected for bone densitometry. Both doses of the peptide resulted in significant increases in bone density as determined by pQCT. Young adult rats were injected locally with a single dose of the peptide (1 μg) or saline into the marrow cavity of the distal femur. One week after the single injection, the bones were collected for radiographic and histological evaluation. The saline controls showed no evidence of bone formation, whereas the peptide treated animals demonstrated bone development at the injection site. These data suggest that DBP-MAF and the synthetic peptide represent therapeutic opportunities for the treatment of a number of bone diseases and skeletal disorders. Systemic administration could be used to treat osteoporosis and a number of other osteopenias and local administration could be effective in fractures, bony defect repairs, spinal surgery and joint replacement.

F515

$1,25(OH)_2D_3$ Synergizes with the PPARγ-Selective Ligand, BRL-49653, to Increase Adipogenesis in Rat Calvaria Cell Cultures. K. Ojzumi, Y. Yoshiko, J. E. Aubin. Anatomy and Cell Biology, University of Toronto, Toronto, ON, Canada.

To investigate the effect of $1,25(OH)_2D_3$ on the conversion of osteoprogenitor cells into adipocytes, rat calvaria (RC) cells were treated with $1,25(OH)_2D_3$ and/or BRL-49653, a potent PPARγ-selective ligand. The expression of PPARs and C/EBPs, which are two transcription factor families that regulate adipocyte differentiation, was also assessed. As reported previously, $1,25(OH)_2D_3$ induced adipocyte colonies and adipocyte marker expression, while completely inhibiting bone nodule formation and the expression of most osteoblast markers in RC cultures; an exception was that $1,25(OH)_2D_3$ increased OPN expression at early culture stages. Although the inverse relationship between osteoblast and adipocyte marker expression and osteoblast and adipocyte colony formation suggested the conversion of osteoprogenitor cells into adipocytes, the number of adipocyte colonies in $1,25(OH)_2D_3$-treated dishes was much less than the number of bone colonies/nodules in vehicle-treated dishes. This apparent discrepancy in fate redirection of osteoprogenitors to adipocytes was altered when RC cells were subjected to combined treatment with $1,25(OH)_2D_3$ and BRL-49653, which induced a large number of mature adipocyte colonies, suggesting that $1,25(OH)_2D_3$ has dual roles: inducing adipocyte maturation in some preadipocytes and inducing osteoprogenitor cells to select an adipocyte fate that is then completed in response to the PPARg selective ligand. Although both $1,25(OH)_2D_3$ and BRL-49653 increased PPARγ and C/EBPα expression, BRL-49653 had no effect on osteoblast differentiation. However, our data support the hypothesis that the inhibitory effect of $1,25(OH)_2D_3$ on osteoblast differentiation is based on its induction of C/EBPδ, which is induced earlier than PPARγ during initiation of adipogenesis. The present study suggests that committed osteoprogenitor cells in RC cell cultures are redirected in fate choice by $1,25(OH)_2D_3$ but undergo marked conversion into mature adipocytes only after combination treatment with $1,25(OH)_2D_3$ and the PPARγ selective ligand.

F517

Phosphorylation of the Human Vitamin D Receptor by Protein Kinase A Downregulates $1,25(OH)_2D_3$-dependent Transactivation by Reducing Retinoid X Receptor β Heterodimerization. J. C. Hsieh, H. T. L. Dang,* M. A. Galligan,* G. K. Whitfield, P. W. Jurutka, P. D. Thompson, C. A. Haussler,* M. R. Haussler. Biochemistry & Molecular Biophysics, College of Medicine, University of Arizona, Tucson, AZ, USA.

Phosphorylation of the human vitamin D receptor (hVDR) includes protein kinase C (PKC) action at serine-51 and casein kinase-II (CK-II) phosphorylation of serine-208, posttranslational modifications that attenuate and potentiate receptor activity, respectively. Preliminary work from our laboratory suggested that protein kinase A (PKA) can also phosphorylate hVDR between amino acids 133 and 201. To elucidate the exact PKA phosphorylation site(s) of hVDR, a series of C-terminally truncated mutants (δ134, δ180, δ190 and δ202) were expressed in transfected COS-7 cells, immunoprecipitated with VDR antibody, and incubated with PKA and [^{32}P]ATP in vitro. Visualization of these reactions by SDS-PAGE indicated that the major PKA phosphorylation site of hVDR is localized between residues 180-190, a region that contains a cluster of four consecutive serines, ^{182}Ser-Ser-Ser-Ser^{185}, and a single serine at position 187. These serines were individually mutated to alanine using δ190 hVDR, the native receptor, and S51A/S208A (to eliminate PKC and CK-II sites) as templates, and the resulting mutant hVDRs were tested for their ability to serve as PKA substrates, in vitro. The results showed that the S182A mutant hVDR was least able to serve as a PKA substrate. Furthermore, when intact transfected COS-7 cells were treated with [^{32}P]orthophosphate, the S182A mutant displayed the largest reduction in phosphorylation compared to the other alanine-substituted hVDRs. We therefore conclude that serine-182 is a primary PKA phosphorylation site in hVDR, both in vitro and in vivo. As a test of the functional consequence of this phosphorylation event, an aspartate-substituted mutant (S182D) was created to mimic the negative charge of a phosphorylated serine. Utilizing the mammalian two-hybrid assay, it was observed that, while the S182A mutant could associate normally with the retinoid X receptor-β (RXRβ) dimeric partner, S182D was significantly impaired in this interaction. Also, in cotransfection assays with a $1,25(OH)_2D_3$-responsive reporter gene, S182A hVDR exhibited normal transactivation, but the S182D mutant possessed only 50% of wild-type hVDR activity. Taken together, these observations strongly suggest not only that serine-182 can be a target of PKA phosphorylation in hVDR, but that this posttranslational event may significantly inhibit hVDR dimerization with RXRβ, thereby attenuating the ability of hVDR to mediate $1,25(OH)_2D_3$-dependent transactivation of target genes.

APPENDIX C

A Novel Anabolic Peptide Activates Osteogenic Gene Expression in Rat Stromal Cells

Gary B. Schneider[1], Kristina J. Grecco[2*], Denise McBurney[2*], Walter E. Horton[2*]

[1]Weill Cornell Medical College in Qatar, New York NY

[2]Northeastern Ohio Universities College of Medicine, Rootstown OH

ABSTRACT

Two weeks of intermittent subcutaneous injections of short peptides from the third domain of the human serum vitamin D binding protein (DBP) to intact, adult rats results in significant increases in total density and strength of long bones. We tested whether these peptides would act directly on differentiated osteoblasts or less differentiated stromal cells derived from femurs of post-natal rats. The cells were treated with PTH (5 nMolar) or 5ng/ml of either peptide 10 or peptide 12 (10 and 12 amino acid fragments of DBP) for time periods ranging from 24 to 72 hours. Total RNA was extracted, reverse transcribed into cDNA, and the relative expression level of a group of marker genes was determined by quantitative real time pcr (QtRT-PCR) normalized to 18S RNA. Peptide 10 induced the up-regulation of mRNA coding for Alkaline Phosphatase (2.3-fold), Collagen I (3-fold), Osteocalcin (17-fold), and Osteopontin (4.7-fold) by 72 hours of treatment. An independent experiment showed a similar pattern of induction at the 48-hour time point and indicated an up-regulation of PCNA mRNA suggesting a proliferative response. PTH and peptide 12 induced a more variable and less robust pattern of osteogenic gene expression. Peptide 10 also induced the expression of these genes in osteoblast cells but the response was slower and less dramatic. These results suggest that the relatively undifferentiated stromal cells present in the marrow may be the target for the anabolic effects of the DBP peptides.

VITAMIN D BINDING PROTEIN

- DBP is a member of the _2-globulin family of serum proteins
- Produced in the liver and secreted into the blood
- 458 amino acids, divided into 3 domains
- Glycosylated in the third domain

PEPTIDE CHARACTERISTICS

- Synthetically produced
- Based on human amino acid sequence near the site of glycosylation in the third domain of DBP
- Fragments 3 to 14 amino acids in length
- Novel peptide - no homologies other than DBP

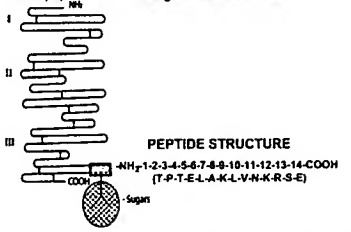

Figure 1 – DBP Protein and Peptide Structure. All peptide fragments were generated by deletions of amino acids from the C-terminal end. The number on the figures represent the number of amino acids in each fragment starting at the N-terminal. All fragments contain the potential glycosylation site on the native protein.

IN VIVO STUDY DESIGN

- S.C. injections of saline or peptide (0.4 ng/g body weight) were given every other day for 2 weeks
- Two days after the final injections, rats were sacrificed
- Femurs and tibiae were collected for bone densitometry

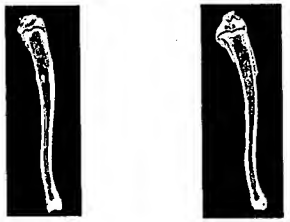

Figure 2 – CT Scans of Bone. These illustrated scans are midline longitudinal slices and represent control and treated adult rats.

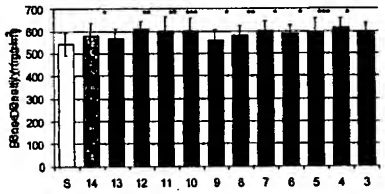

Figure 3 – Total Bone Density. Animals treated with the various peptide fragments demonstrated a range of responses from highly significant increases in bone density to no change. * = p<0.05, ** = p<0.01, *** = p<0.001.

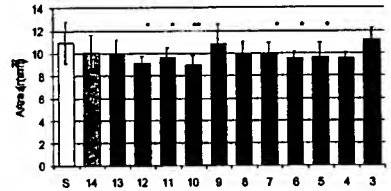

Figure 4 – Total Area. Animals treated with the various peptide fragments demonstrated a range of responses from significant reductions in cross-sectional area of the proximal tibia to no change. * = p<0.05, ** = p<0.01.

BIOMECHANICS DESIGN

- Tibiae were tested to failure in three-point bending.
- The ultimate strength was calculated from the load deflection data and cross-sectional geometries of the mid-shaft.

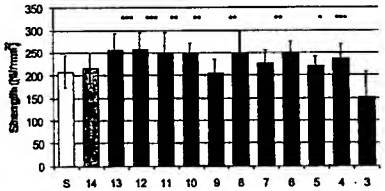

Figure 5 – Bone Strength. Animals treated with the various peptide fragments demonstrated a range of responses from highly significant increases in strength to reductions in strength. * = p<0.05, ** = p<0.01, *** = p<0.001.

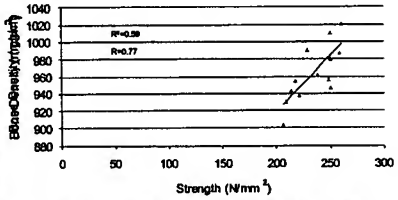

Figure 6 – Bone Density vs. Strength. When bone density was plotted vs. strength there was a highly significant, direct correlation. Those peptide treatments resulting in the most significant increases in bone density in the tibial mid-shaft were also the strongest. p=0.002

IN VITRO STUDY DESIGN

- Stromal cells and osteoblasts were collected from post-natal rat femurs
- Cells were treated with PTH, ABP10, or ABP12 for 24-72 hrs.
- Relative expression of 5 genes (Alkaline Phosphatase, collagen Type 1, Osteocalcin, Osteopontin, and PCNA) was determined by QtRT-PCR normalized to 18s RNA

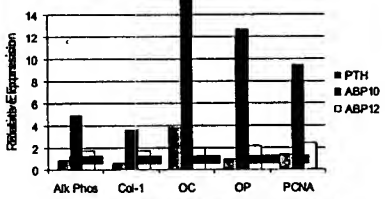

Figure 7 – Stromal Cells after 48 hrs. of treatment.

(Dashed line indicates relative gene expression at a similar level between control and treated cells)

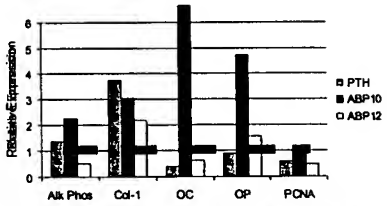

Figure 8 – Stromal Cells after 72 hrs. of treatment.

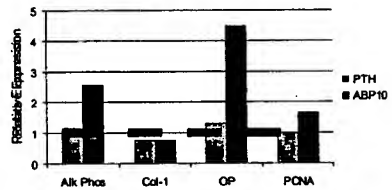

Figure 9 – Osteoblast cells after 24 hrs. of treatment.

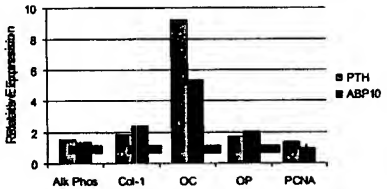

Figure 10 – Osteoblast cells after 72 hrs. of treatment.

CONCLUSIONS

- The intermittent injections of a number of the peptide fragments significantly enhance bone density in the long bones of adult rats.
- A major contributor to the increase in total bone density was highly significant increases in cortical/subcortical density.
- Those treatment groups demonstrating the most significant increases in total bone density showed decreases in cross-sectional area of the tibial bones.
- The various peptide fragments had differential effects on bone strength, ranging from highly significant increases in strength to decreases in strength.
- There was a strong correlation between bone density and bone strength among the treatment groups. Those treatments which enhanced bone density also resulted in stronger bones–suggesting better bone quality.
- *In vitro* data suggest that the peptides can activate a bone pattern of gene expression in stromal osteoblastic precursor cells

APPENDIX D

pQCT Analysis of the Anabolic Effects of a Group of Novel Small Peptides on Bone in Intact Adult Rats

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Division of Basic Medical Sciences, Northeastern Ohio Universities College of Medicine, Rootstown, OH

ABSTRACT

We have previously demonstrated that 14 amino acid peptide fragments, based on a human sequence from the third domain of the native serum protein, vitamin D binding protein (DBP) elicit anabolic effects on the skeleton. Intermittent systemic injections of the peptide to intact, newborn and adult rats resulted in significant increases in total bone density within two weeks of treatment. A single, local injection of the same 14 a.a. peptide generated new bone at the site of injection at one week (Schneider et. al., JBMR, 16:S231). In the current study we evaluated peptide fragments ranging in length from 3-13 a.a. from the same region of the DBP protein in intact adult rats. Female rats were given subcutaneous injections of saline or peptide (0.4 ng/g body wt.) every other day for two weeks; two days after the final injections, the rats were euthanized and the femurs and tibias collected for analysis by peripheral quantitative computerized tomography (pQCT). Specific slices through the metaphysis and mid-shaft of each bone were analyzed from the scans. The proximal tibia metaphysis and mid-shaft slices were representative of the skeletal responses to the various peptide fragments. The efficacy of the peptide fragments was based on their effect on total bone density. Fragments 12, 11, 10, and 4 amino acids in length demonstrated highly significant ($p < 0.001$) increases in total bone density in the proximal tibia metaphysis. All of these peptides also illustrated the same trends with respect to the other parameters evaluated: increases in cortical/subcortical bone density, decreases in total surface area and decreases in periosteal and endosteal circumferences. The effective peptides also elicited significant increases in bone density of the mid-shaft, but the other trends were not as prevalent. Those peptide fragments that did not increase bone density did not illustrate any consistent trends with respect to the other parameters measured. The anabolic peptides appear to be increasing bone density and decreasing the diameter and cross-sectional area of the bone. None of the peptides affected bone length, which remained consistent with the saline controls. We hypothesize that the increased bone density may be reflecting better quality bone; the decrease in cross-sectional surface area may be in response to this superior quality bone. Biomechanical testing of the bones treated with the effective peptides is planned to test this hypothesis.

VITAMIN D BINDING PROTEIN

- DBP is a member of the α2-globulin family of serum proteins
- Produced in the liver and secreted into the blood
- 458 amino acids, divided into 3 domains
- Glycosylated in the third domain

PEPTIDE CHARACTERISTICS

- Synthetically produced
- Based on human amino acid sequence near the site of glycosylation in the third domain of DBP
- Fragments 3 to 14 amino acids in length
- Novel peptide - no homologies other than DBP

EXPERIMENTAL DESIGN (ADULTS)

- S.C. injections of saline or peptide (0.4 ng/g body weight) were given every other day for 2 weeks
- Two days after the final injections, rats were sacrificed
- Femurs and tibiae were collected for bone densitometry

Figure 1 – CT Scans of Bone. These illustrated scans are midline longitudinal slices and represent control and treated adult rats.

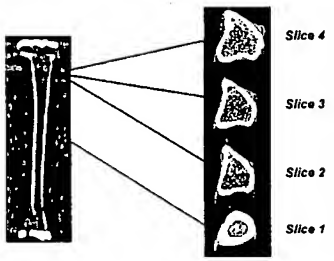

Figure 2 - pQCT Analyses. The image on the left is a longitudinal "scout" view of the tibia of a young adult rat. All analyses of bone density included three slices from the proximal tibial metaphysis and a single mid-shaft slice (Slice 1).

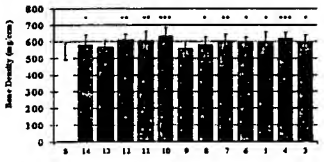

Figure 3 - Total Bone Density. Animals treated with the various peptide fragments demonstrated a range of responses from highly significant increases in bone density to no change. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

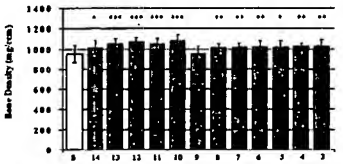

Figure 4 – Cortical/Subcortical Bone Density. Animals treated with the various peptide fragments demonstrated a range of responses from highly significant increases in bone density to no change. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

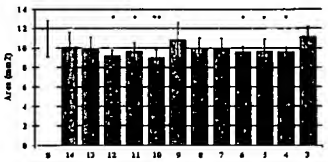

Figure 5 – Total Area. Animals treated with the various peptide fragments demonstrated a range of responses from significant reductions in cross-sectional area of the proximal tibia to no change. * = $p<0.05$, ** = $p<0.01$.

DBP PROTEIN STRUCTURE

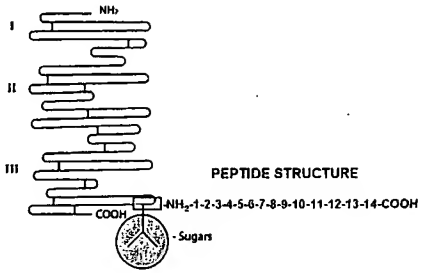

Figure 6 – DBP Protein and Peptide Structure. All peptide fragments were generated by deletions of amino acids from the C-terminal end. The number on the figures represent the number of amino acids in each fragment starting at the N-terminal. All fragments contain the potential glycosylation site on the native protein.

CONCLUSIONS

- The intermittent injections of a number of the peptide fragments significantly enhanced bone density in adult rats. Specifically, fragments 12, 11, 10, 7, and 4 effectively increased bone density.
- The major contribution to the increase in total bone density was highly significant increases in cortical/subcortical bone density.
- Those treatment groups demonstrating the most significant increases in total bone density demonstrated decreases in the cross-sectional area of the tibial slices.
- We hypothesize that increased bone density may be reflecting better bone quality (strength) and the decrease in cross-sectional surface area of the effected bones may be in response to superior bone quality.
- Preliminary biomechanical testing (3-point bending) suggests that those peptide treatments eliciting increased bone density also generated stronger bones. There was a strong correlation between bone density and bone strength among the treatment groups.

APPENDIX E

Proceedings of IMECE'03
2003 ASME International Mechanical Engineering Congress & Exposition
Washington, D.C., November 16-21, 2003
IMECE2003-43044

EFFECT OF PHARMACEUTICAL BONE GROWTH STIMULATION WITH NOVEL ANABOLIC PEPTIDES: BIOMECHANICAL AND BONE DENSITY MEASUREMENTS IN A RAT MODEL

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ABSTRACT

Pharmaceutical bone growth stimulation holds promise for prevention and treatment bone disorders, and the enhancement of fracture healing. Bone growth hormones have begun to have limited clinical use, but can illicit adverse side effects. Recent studies have shown that short peptides (less than 15 amino acids) derived from the protein sequence of Vitamin D Binding Protein (DBP), can enhance bone formation (osteogenesis). These peptides may have potential as controllable bone growth stimulators without the adverse side effects and cost of bone growth hormones. Rats, injected every other day for two weeks with DBP-based peptide fragments ranging from 3 to 13 amino acids in length, were euthanized and the tibias and femurs were scanned by peripheral quantitative computerized tomography (pQCT) to determine bone density and cross-sectional geometric properties. The bones were then tested in three-point bending to determine strength and bending modulus. Injection of DBP-based peptides over only a 2-week period resulted in significant ($p<0.05$) increases in bone density and material properties in the experimental rat bones in comparison to controls injected with saline. The short length of these effective peptides suggests their use not only in systemic injections but also as clinically convenient pills taken orally for pharmaceutically induced bone growth stimulation.

INTRODUCTION

Pharmaceutical bone growth stimulation holds promise for the prevention and treatment bone disorders such as osteoporosis, osteopenia, osteopetrosis and osteogenesis imperfecta, and for the enhancement of fracture healing and spinal fusion. Suppressors of bone resorption and bone turnover such as bisphosponates, which inhibit osteoclastic action, are now widely used to treat osteoporosis (Brunelli and Einhorn, 1998). The anabolic capabilities of bone growth hormones, which stimulate osteoblastic activity and bone growth, have begun to be exploited to enhance bone consolidation for spinal fusion (Khan, et al., 2002). However, their current clinical use is as yet quite limited. A drawback of the general use of bone growth hormones is their high processing cost and the risks of unintended and adverse side effects that accompany their use (Brunelli and Einhorn, 1998, Khan, et al., 2002).

Recent studies of osteopetrosis, a rare and usually fatal disease characterized by an abnormal increase in bone density, have shown that some forms of this disease may be the result of a defect in the biochemical pathway that leads to the expression of human serum protein, Vitamin D Binding Protein (DBP). This observation has lead to investigation of the potential bone density enhancing capability of DBP. Indeed, it has now been demonstrated that DBP has anabolic effects on bone (Schneider, et al., 2001). It has also been shown that a 14 amino acid peptide fragment from the third domain of the protein sequence of DBP also has anabolic capabilities (Schneider, et al., 2002). The short amino acid length of this anabolic fragment invites further investigation of such fragments because of their considerable utility in oral medications to treat bone density reducing diseases. The purpose of this study was to assess changes in bone density and mechanical properties resulting from injection of peptide fragments, 3 to 13 amino acids long, created by single amino acid deletions from the previously studied 14 amino acid peptide derived form the protein sequence of DBP.

METHODS

One hundred and twenty-seven, adult, genetically intact, female rats, 7 to 8 weeks old and weighing a nominal 180 g, were involved in these tests. Ninety-nine of the animals were randomly assigned to 11 experimental groups. Each experimental group consisted of 9 animals that were injected subcutaneously every other day for two weeks with one of the tested peptides (0.4 ng/g body weight per injection). The remaining 28 animals formed the control group, which received injections of saline on the same schedule. All animal testing in this study was carried out with prior approval of the Institutional Animal Care and Use Committee of the Northeastern Ohio Universities College of Medicine.

Two days after their final injections, the animals were euthanized, and their lower extremity bones were harvested. The left femur and tibia of each animal were stripped of all soft tissue and were scanned by peripheral quantitative computerized tomography (pQCT: Norland Stratec XCT Small Animal Bone Densitometer) to determine bone density and to determine mid-shaft cross-sectional geometric properties, principally, the area moments and products of inertia. The bones were then stored frozen in alcohol prior to mechanical testing.

The specimens were tested to failure in three-point bending at a displacement rate of 5 mm/min on a materials testing system (Model 812, MTS, Minneapolis, MN). The maximum load and stiffness were determined from the load-deflection curve recorded digitally during the bending test. The ultimate strength (stress) and the bending modulus were calculated from the load-deflection data and the previously determined cross-sectional geometries using standard formulas appropriate for 3-point bending of a simply-supported beam.

The bone density and mechanical property results of the experimental groups were statistically compared to those of the control group by ANOVA, followed by SNK multiple range tests for significance ($p<0.05$).

RESULTS

For both the tibias and femurs, the calculated bending moduli for most of the experimental groups were greater than that of the control group, but their variances were too large to allow any of the differences to be statistically significant. For the tibias, several of the experimental groups demonstrated significantly increased (*$p<0.05$) bending strength, Figure 1. The calculated bending strengths of the femurs did not differ among the tested groups.

Total bone densities for both the femurs and tibias were greater than control in most of the experimental groups, as seen in Figure 2 for the tibias. The increase was significant (*$p<0.05$) for some of the groups. There was a significant correlation between total bone density and bending strength ($R = 0.77$, $p<0.01$), Figure 3. There were also significant increases in cortical and subcortical bone density, but there were no changes in trabecular bone density. The periosteal and endosteal circumferences tended to be smaller in the experimental groups than in the control group, but the changes were not significant.

DISCUSSION

This study involved a limited number of test animals per experimental group and employed a short dosing period of only two weeks. The significant increases in bone density and strength that were seen indicate the potential of short amino acid peptides derived for the human serum protein, Vitamin D binding protein as bone density increasing pharmaceuticals. Additional studies with larger numbers of animals exposed to longer dosing periods are warranted.

REFERENCES

Brunelli, M.P., and Einhorn, T.A., 1998, "Medical Management of Osteoporosis. Fracture Prevention," Clin. Orthop., 348, 15-21.

Khan, S.N., Sandhu, H.S., Lane, J.M., Cammisa, F.P. Jr, Girardi, F.P., 2002, "Bone Morphogenetic Proteins: Relevance in Spine Surgery." Orthop Clin North Am. 33(2):447-63.

Schneider, G.B., Grecco, K.J., Safadi, F.F., and Popoff, S.N., 2001, "The Anabolic Effect of Vitamin D Binding Protein-Macrophage Activating Factor (DBP-MAF) and a Novel Small Peptide on Bone.", J. Bone Min. Res. 16(Suppl 1) S231.

Schneider, G.B., Bui, D.T., and Grecco, K.J., 2002, "pQCT Analysis of the Anabolic Effects of a Group of Novel Small Peptides on Bone in Intact Adult Rats", J. Bone Mineral Res., 17 (Suppl 1) S377.

ACKNOWLEDGEMENT

This work was supported, in part, by The National Institutes of Health, Grant #DE06065, The Summa Health System Foundation, and the Robertson-Hoyt Fund.

FIGURE 1:

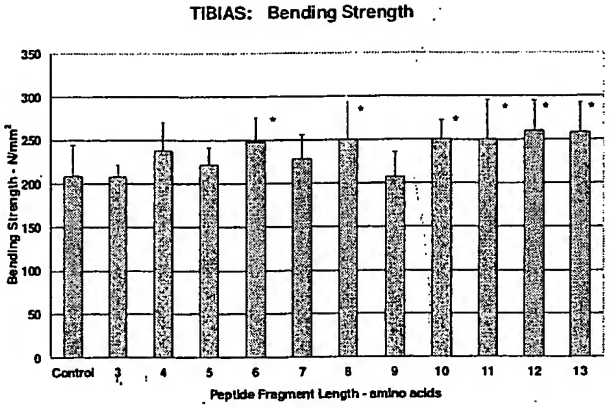


FIGURE 2:

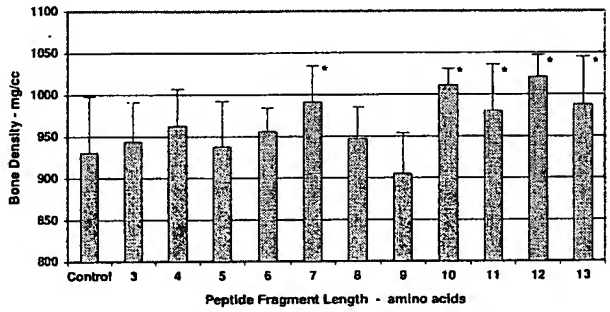
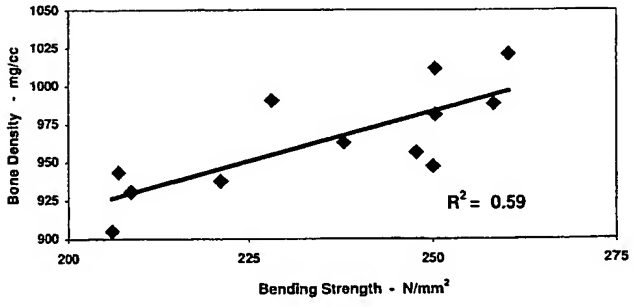


FIGURE 3:



APPENDIX F

The Anabolic Effect of Vitamin D Binding Protein-Macrophage Activating Factor (DBP-MAF) and a Novel Small Peptide on Bone

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[2]Department of Anatomy and Cell Biology, Temple University School of Medicine, Philadelphia, PA.

ABSTRACT

Vitamin D binding protein-macrophage activating factor (DBP-MAF) has previously been shown to stimulate bone resorption and correct the skeletal defects associated with osteopetrosis in two non-allelic mutations in rats. This same protein and a small fragment of the protein have now been shown to demonstrate an anabolic effect on the skeleton of both newborn and young adult, intact rats. The novel peptide fragment was synthetically produced based on the human amino acid sequence at the site of glycosylation in the third domain of the native protein (DBP). The peptide tested is 14 amino acids in length and demonstrates no homologies other than to that region of DBP. Newborn rats were injected i.p. with saline, peptide (0.4 ng/g body wt.) or DBP-MAF (2 ng/g body wt.) every other day from birth to 14 days of age. On day 16 the rats were euthanized and the long bones collected for bone densitometry by pQCT. Serum was collected for evaluation of osteocalcin levels as an indication of bone formation and urine was analyzed for deoxypyridinoline (Dpd) as a measure of bone resorption. After two weeks of treatment with either the whole protein (DBP-MAF) or the small peptide, bone density was significantly increased in the treated animals compared to the saline controls. Serum osteocalcin levels were significantly enhanced and Dpd levels in the urine were significantly decreased in the protein and peptide treated animals. Young adult female rats were given s.c. injections of saline or peptide (0.4 ng/g body wt. or 5 ng/g body wt.) every other day for two weeks; two days after the final injections, the rats were euthanized and the femurs and tibias collected for bone densitometry. Both doses of the peptide resulted in significant increases in bone density as determined by pQCT. Young adult rats were injected locally with a single dose of the peptide (1 µg) or saline into the marrow cavity of the distal femur. One week after the single injection, the bones were collected for radiographic and histological evaluation. The saline controls showed no evidence of bone formation, whereas the peptide treated animals demonstrated bone development at the injection site. These data suggest that DBP-MAF and the synthetic peptide represent therapeutic opportunities for the treatment of a number of bone diseases and skeletal disorders. Systemic administration could be used to treat osteoporosis and a number of other osteopenias and local administration could be effective in fractures, bony defect repairs, spinal surgery and joint replacement

VITAMIN D BINDING PROTEIN

- DBP is a member of the α2-globulin family of serum proteins
- Produced in the liver and secreted into the blood
- 458 amino acids, divided into 3 domains
- Glycosylated in the third domain

CONVERSION OF DBP TO DBP-MAF

- Conversion mediated by β-galactosidases
- Conversion involves the cleavage of sugars from the third domain of the protein
- DBP-MAF has effects on both the immune and skeletal systems
- DBP and/or cascade to produce DBP-MAF is defective in animal models of osteopetrosis

PEPTIDE CHARACTERISTICS

- Synthetically produced
- Based on human amino acid sequence at the site of glycosylation
- 14 amino acids or less
- Novel peptide - no homologies other than DBP
- Demonstrates anabolic effect on skeletal system, but no influence on immune system
- Novel therapeutic application, not an anti-resorptive, rather an anabolic agent

EXPERIMENTAL DESIGN (NEWBORNS)

- I.P. injections of saline, peptide (0.4 ng/g body weight) or DBP-MAF (2 ng/g body weight) were given every other day from birth to 14 days
- On day 16, rats were sacrificed
- Femurs and tibiae were collected for bone densitometry

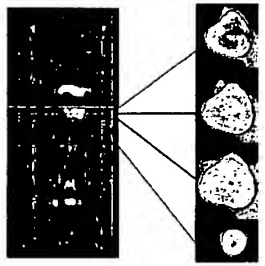

Figure 1 - pQCT Analyses. The image on the left is a longitudinal "scout" view of the tibia of a 16-day-old rat. All analyses of bone density included three slices from the proximal tibial metaphysis and a single mid-shaft slice (number 4).

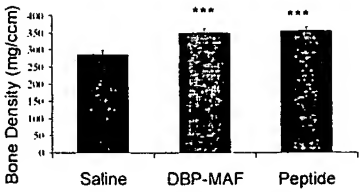

Figure 2 - Total Bone Density (Newborns). The total bone density of the proximal tibial metaphysis (slice 2) was highly significantly increased in animals treated with the whole protein (DBP-MAF) and a glycosylated form of the peptide as compared to the saline-treated controls. *** = $p < 0.001$

EXPERIMENTAL DESIGN (ADULTS)

- S.C. injections of saline or peptide (0.4 ng/g body weight or 5 ng/g body weight) were given every other day for 2 weeks
- Two days after final injections, rats were sacrificed
- Femurs and tibiae were collected for bone densitometry

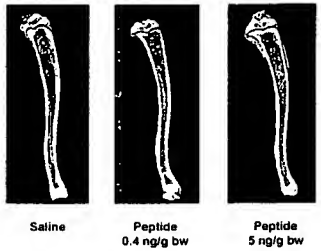

Figure 3 – CT Scans of Bone. The three illustrated scans are midline longitudinal slices from control and treated adult rats. The increase in amount of bone and increase in density was most pronounced at the higher concentration of peptide (5ng/g body wt.)

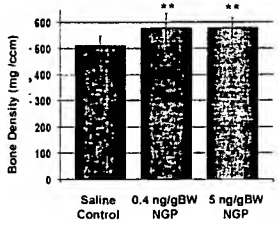

Figure 4 – Total Bone Density (Adults). Animals treated with two different concentrations of the non-glycosylated peptide (NGP) demonstrated very significant increases in total bone density as compared to controls. ** = $p < 0.01$

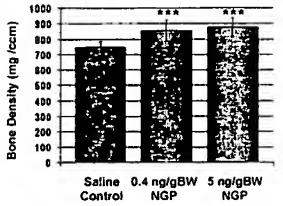

Figure 5 – Cortical Bone Density (Adults). Animals treated with two different concentrations of the non-glycosylated peptide (NGP) demonstrated highly significant increases in cortical bone density as compared to controls. *** = $p < 0.001$.

LOCAL INJECTION OF PEPTIDE

- Young adult rats were injected into the distal femur with 1 µg of peptide or saline
- One week after the single injection, the bones were collected for radiographic and histologic evaluation

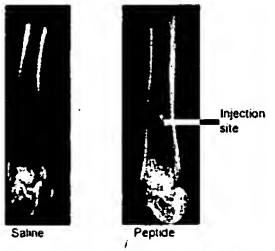

Figure 6 – X-Rays of Local Injection. Radiographs of the distal femur of adult rats injected with a single dose of peptide (right) or saline (left). Radio opacity of the injection site indicates osteoinduction in the bone marrow cavity. Bone density is also increased in the area surrounding the injection site indicating osteogenesis.

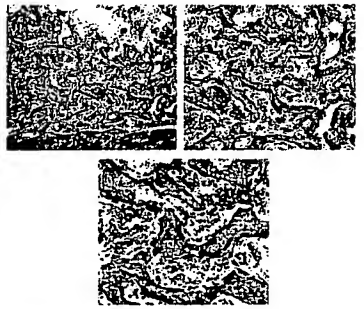

Figure 7 – Goldner Trichrome Stained Sections. Micrographs of sections from the peptide injection site stained with Goldner trichrome. All three magnifications demonstrate extensive woven bone formation in the bone marrow cavity.

CONCLUSIONS

- The intermittent injections of both the native DBP-MAF protein and the small peptide fragment significantly enhanced bone density in young rats.
- The intermittent injections of both glycosylated and non-glycosylated peptide fragments of DBP-MAF significantly enhanced bone density in adult rats.
- The local injection of a single dose of the peptide fragment into the bone marrow of the femur resulted in both osteoinduction and osteogenesis in the region of the injection site within one week.
- These findings suggest therapeutic opportunities for the systemic administration of these agents to treat disorders such as osteoporosis, osteogenesis imperfecta, osteopenia associated with cancer, dialysis or steroid therapy and space travel.
- The local administration of these agents could be used therapeutically for fracture repair, bony defects, spinal surgery and joint replacement.

APPENDIX G

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The Anabolic Effects of Vitamin D-Binding Protein-Macrophage Activating Factor (DBP-MAF) and a Novel Small Peptide on Bone

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ABSTRACT: Vitamin D-binding protein-macrophage activating factor (DBP-MAF) has previously been shown to stimulate bone resorption and correct the skeletal defects associated with osteopetrosis in two nonallelic mutations in rats. This same protein and a small fragment of the protein have now been shown to demonstrate an anabolic effect on the skeleton of both newborn and young adult, intact rats. The novel peptide fragment was synthetically produced based on the human amino acid sequence at the site of glycosylation in the third domain of the native protein (DBP). The peptide tested is 14 amino acids in length and demonstrates no homologies other than to that region of DBP. Newborn rats were injected i.p. with saline, peptide (0.4 ng/g body wt.) or DBP-MAF (2 ng/g body wt.) every other day from birth to 14 days of age. On day 16 the rats were euthanized and the long bones collected for bone densitometry by pQCT. After 2 weeks of treatment with either the whole protein (DBP-MAF) or the small peptide, bone density was significantly increased in the treated animals compared to the saline controls. Young adult female rats (180 grams) were given s.c. injections of saline or peptide (0.4 ng/g body wt. or 5 ng/g body wt.) every other day for 2 weeks; 2 days after the final injections, the rats were euthanized and the femurs and tibias collected for bone densitometry. Both doses of the peptide resulted in significant increases in bone density as determined by pQCT. Young adult rats were injected locally with a single dose of the peptide (1 μg) or saline into the marrow cavity of the distal femur. One week after the single injection, the bones were collected for radiographic and histological evaluation. The saline controls showed no evidence of new bone formation, whereas the peptide-treated animals demonstrated osteoinduction in the marrow cavity and osteogenesis of surrounding cortical and metaphyseal bone. These data suggest that DBP-MAF and the synthetic peptide represent therapeutic opportunities for the treatment of a number of bone diseases and skeletal disorders. Systemic administration could be used to treat osteoporosis and a number of other osteopenias, and local administration could be effective in fractures, bony defect repairs, spinal surgery, and joint replacement.

KEYWORDS: osteogenesis, osteoinduction, osteoporosis, osteopenia, bone formation

I. INTRODUCTION

Vitamin D-binding protein (DBP) is a serum protein that, through a series of enzymatic cleavages, can be converted to an immune system regulator, vitamin D-binding protein-macrophage activating factor (DBP-MAF) (Yamamoto and Homma, 1991). The DBP, also known as group-specific component, has one vitamin D-binding site in the first domain of the protein that binds vitamin D metabolites in plasma (Haddad and Walgate, 1976), but it also has the ability to bind actin and a number of other agents with equal affinities (Van Baelen et al., 1980; Haddad, 1982). The human DBP has a molecular weight of approximately 58,000 and can be divided into three domains. The third domain (at the c-terminus of the molecule) contains an important glycosylation site. This O-linked glycosylation contains sugar residues that can be cleaved by an inducible β-galactosidase produced by B lymphocytes and an inducible sialidase produced by

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T lymphocytes in response to inflammation (Yamamoto and Homma, 1991; Viau et al., 1983; Yamamoto and Kumashiro, 1993). When this modification of the protein occurs, the resulting molecule becomes a potent activator of macrophages, DBP-MAF.

A relationship between DBP-MAF and the skeletal system has been established based on a series of experiments involving osteopetrotic mutant rodents. Osteopetrosis represents a heterogeneous group of bone disorders characterized by an increase in skeletal mass and a variety of defects associated with the immune system (Popoff and Schneider, 1996; Schneider et al, 1998). Two nonallelic mutations in the rat, osteopetrosis (*op*) and incisors absent (*ia*) demonstrate independent defects in the cascade involved in the inflammation-primed conversion of DBP to DBP-MAF (Yamamoto et al., 1994). *Ex vivo*–generated human DBP-MAF corrects these macrophage defects in both mutations (Popoff and Schneider, 1994). Because this macrophage activator could also potentially play a role in the pathogenesis of the osteoclast dysfunction in these two mutations, the effects of DBP-MAF on the skeletal system was evaluated. Newborn *ia* and *op* rats were treated for 2 weeks with human DBP-MAF, and a number of skeletal parameters were evaluated. DBP-MAF–treated *op* rats had increased numbers of normal-looking osteoclasts and reduced bone volume. The treated *ia* rats had enlarged marrow cavities and normal-looking osteoclasts, which demonstrated normal levels of superoxide production (Schneider et al., 1995). These studies demonstrated that the skeletal defects in these mutations could be improved with exogenous DBP-MAF.

The above findings led to a series of experiments to help establish the mechanism by which the DBP-MAF was influencing the skeletal system. The most obvious explanation was that, as its name implies, the vitamin D-binding protein was carrying vitamin D metabolites that were influencing the bone cells. On the basis of the quantities (picograms per whole animal) of DBP-MAF that elicited a skeletal response in the osteopetrotic mutants, this did not appear to be a viable explanation. *In vitro* studies of osteoclastic activity in the presence of numerous forms of DBP-MAF confirmed this point. Osteoclast activation was the same whether or not the vitamin D-binding site of the DBP-MAF was occupied (Swamy et al., 2001). This study further suggested that the influence on the skeletal system by DBP-MAF most likely resided in the region of the native protein conversion to DBP-MAF—at the glycosylation site in the third domain of the protein.

The *in vitro* studies cited above (Swamy et al., 2001) included dose/response evaluations. The dose of DBP-MAF that elicited the greatest bone resorbing activity *in vitro* was subsequently used in an *in vivo* study of newborn *ia* and normal rats. Contrary to the results of our first study involving the treatment of *ia* rats, which demonstrated enhanced bone resorption, the higher dose of DBP-MAF actually stimulated osteogenesis. This anabolic effect of DBP-MAF in normal newborn rats led to the studies described in this article.

II. MATERIALS AND METHODS

A. Animals

The rats used for the newborn studies described were obtained from a breeding colony at the Northeastern Ohio Universities College of Medicine. This is a breeding colony of wild-type Norway-Hooded rats of the Long Evans strain. These are the wild-type stock of the *ia/ia* mutation. The adult studies were performed on young adult female rats (Charles River Laboratories, Inc., Wilmington, MA. Strain Crl: CD®(SD) IGSBR), which all weighed approximately 180 grams at the onset of the study. All animals were maintained and used according to the principles in the NIH Guide for the Care and Use of Laboratory Animals and the specific guidelines established by the IACUC committees at the Northeastern Ohio Universities College of Medicine and Temple University School of Medicine.

B. Systemic Treatment of Animals

1. Newborn Systemic Studies

Newborn rats, both male and female, were injected intraperitoneally with saline, a 14 amino acid peptide (0.4 ng/g body wt.), or DBP-MAF (2 ng/g

body wt.) every other day from birth until 14 days of age. On day 16, the rats were euthanized and the long bones in the hind limbs were collected for bone densitometry.

2. Adult Systemic Studies

Young adult female rats (180 g.) were given subcutaneous injections of saline or a 14 amino acid peptide (0.4 ng/g body wt. or 5 ng/g body wt.) every other day for 2 weeks; 2 days after the final injections, the rats were euthanized and the femurs and tibias collected for bone densitometry.

C. Analysis of Bones by pQCT

The harvested long bones (femur and tibia) from each animal were stripped of all soft tissue and stored frozen in saline. After thawing, the bones were scanned by peripheral quantitative computerized tomography (pQCT) using a Norland Stratec XCT Research M Bone Densitometer. The standard analysis of each bone included three slices from either the proximal tibial metaphysis or distal femoral metaphysis and a single mid-shaft slice (see Fig. 1). The parameters evaluated from each slice included total bone density, trabecular bone density, cortical/subcortical bone density, and total area.

D. Local Injection Studies

This model has been used to test the anabolic response of other known osteoinductive stimuli, including BMP-2, PGE_2, and CTGF (Li et al., 1995; Safadi et al., 2003). Adult male rats (12–16 weeks of age) were anesthetized and a small area on the dorsal surface of the femur (just distal to midshaft) was exposed surgically through a small skin incision. A tiny hole was made through the cortical bone using a 27-gauge bit on a dental drill and a Hamilton syringe was used to inject a minute volume (20 μL) of saline containing 1 μg of the peptide into the marrow cavity. The hole was immediately plugged using bone wax, the incision was sutured, and animals recovered quickly and uneventfully. Control rats were injected with the same volume of saline or 1% BSA in saline. After 1 week, the animals were euthanized and femurs removed for radiographic and histological analyses.

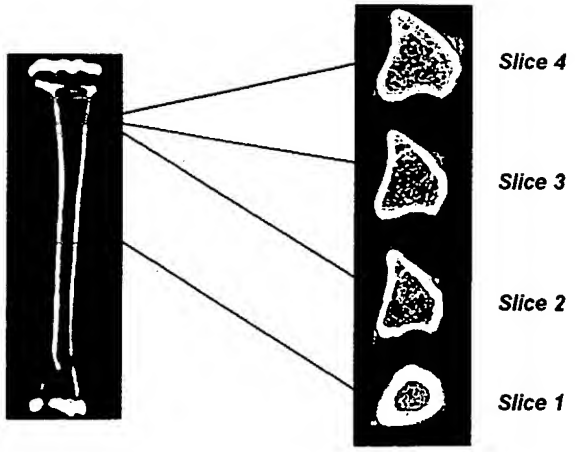

FIGURE 1. This is an illustration of the standard pQCT analysis of tibias and femurs. The image on the left is a longitudinal view of the tibia of a young adult female rat. All analyses of the bones included three slices from the proximal tibial metaphysis and a single mid-shaft slice (slice 1).

E. Reagents

Vitamin D-binding protein (DBP) and DBP-MAF were isolated from human serum via the procedures indicated in Swamy et al. (2001) and were kindly provided by Dr. N. Swamy, Boston University School of Medicine, Boston, MA. The 14 amino acid peptide utilized in these studies was designed based on the amino acid sequence of the human native protein in the immediate vicinity of the glycosylation site in the third domain. Both the glycosylated and nonglycosylated forms of the peptide were synthesized by AnaSpec, San Jose, CA. The amino acid sequence was TPTELAKLVNKRSE. The glycopeptide fragment had an O-linked *N*-acetyl galactosamine attached to the T at a.a. position 3.

F. Statistical Analyses

All of the studies conducted included a minimum of eight rats in each treatment group. Statistical significance was determined using two-tailed, unpaired T tests. Significance was established at $p < 0.05$.

III. RESULTS

A. Newborn Systemic Studies

When the indicated doses of human DBP-MAF or the 14 a.a. glycopeptide were administered to neonatal rats for 2 weeks, there were significant effects on the long bones compared to the vehicle-treated animals. The effects were similar in both male and female animals, and, therefore, the results were merged. The parameters evaluated all demonstrated the same trends in each of the four slices and were seen in both the tibia and femur. The data from slice 4 (see Fig. 1) of the tibia will be presented as representative of the effects of these agents.

Figure 2 illustrates the profound effect both the whole protein (DBP-MAF) and the peptide fragment had on total bone density after 2 weeks of treatment. Both treatments resulted in over a 25% increase in bone density compared to the control animals. The whole protein and peptide fragment performed equally in increasing bone density ($p < .0001$). Increases in trabecular bone density and cortical/subcortical bone density both contributed to the increases illustrated in Figure 2 (data not shown). The cross-sectional area of the proximal tibia also increased significantly in response to both treatments (Fig. 3). The endosteal circumference, periosteal circumference, and cortical thickness all increased in the animals treated with both agents (data not shown). Although the thickness of the bones increased in the treated animals, the overall lengths of the bones was not altered by either treatment (data not shown).

B. Adult Systemic Studies

The treatment of adult female rats with two doses of the glycosylated form of the peptide resulted in significant increases in total bone density (Fig. 4). There was little change in trabecular bone density in the adult animals (data not shown); the majority of the increase in total bone density was due to increases in cortical/subcortical density (Fig. 5). A nonglycosylated form of the same peptide was tested in the adult animals and again demonstrated a significant effect on total bone density (Fig. 6). There was little change in trabecular bone density, and, again, the cortical/subcortical density was the major contributor to the overall change in total density (data not shown). Unlike the neonatal treated rats, the adult animals did not respond to the peptides with an increase in the cross-sectional area of the slices evaluated; the bones did not grow thicker. As was seen in the young animals, none of the treated animals had any change in bone length (data not shown).

C. Local Injection Studies

A single injection of 1 μg of the nonglycosylated form of the peptide into the red bone marrow of

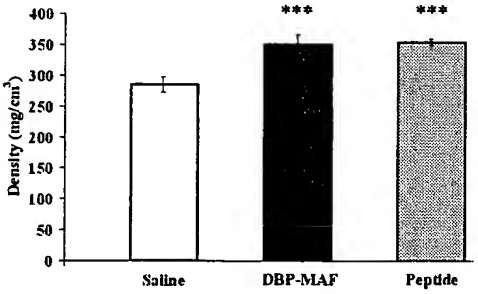

FIGURE 2. Total bone density (newborn study). The total bone density of the proximal tibial metaphysis (slice 3) was highly significantly increased in animals treated with the whole protein (DBP-MAF) and a glycosylated form of the 14 a.a. peptide fragment, as compared to saline-treated controls. Bars represent mean ($n = 8$) ± SD. *** = $p < 0.001$.

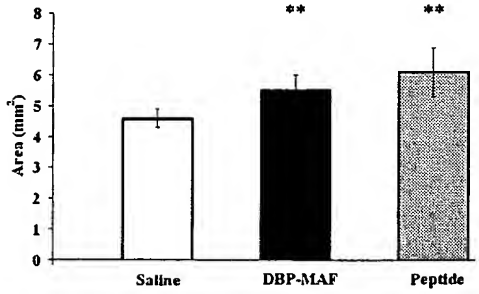

FIGURE 3. Total cross-sectional area (newborn study). The total bone area of the proximal tibial metaphysis (slice 3) was significantly increased in animals treated with the whole protein (DBP-MAF) and the glycosylated form of a 14 a.a. peptide fragment, as compared to saline-treated controls. Bars represent mean ($n = 8$) ± SD. ** = $p < 0.01$, *** = $p < 0.001$.

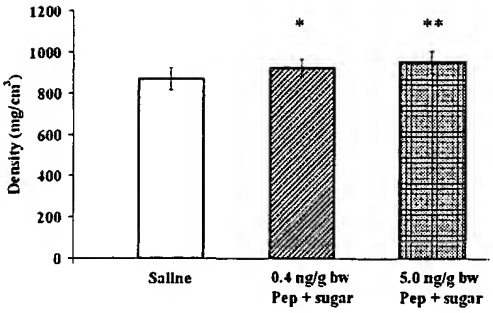

FIGURE 4. Total bone density (adult study). The total bone density of the midshaft of the tibia (slice 1) was significantly increased in animals treated with two doses of the glycosylated form of the 14 a.a. peptide fragment, as compared to saline-treated controls. Bars represent mean (n = 8) ± SD. * = $p < 0.05$, ** = $p < 0.01$.

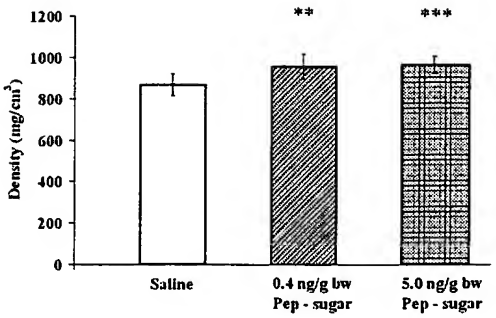

FIGURE 6. Total bone density (adult study). The total bone density of the midshaft of the tibia (slice 1) was significantly increased in animals treated with two doses of the nonglycosylated form of the 14 a.a. peptide fragment, as compared to saline-treated controls. Bars represent means (n = 8) ± SD. ** = $p < 0.01$, *** = $p < 0.001$.

adult rats led to the formation of new bone within seven days. Figure 7 is the X-ray of the injection site from both a peptide- and saline-treated animal. There is no evidence of increased radiopacity in the saline-treated femur, but the peptide-treated bone shows evidence of new bone formation in the proximity of the injection site. Furthermore, the peptide-treated bone demonstrates greater radiopacity in the adjacent cortical bone and trabecular bone in the distal femoral metaphysis. These results suggest that new bone had been formed around the injection site and bone density had been enhanced at the skeletal sites adjacent to the injection site. The saline injection appeared to have no effect on the bone and looked essentially the same as a nontreated bone, radiographically. Histological evaluation confirmed the X-ray findings. Figure 8 illustrates an extensive area of woven bone that was formed in the marrow cavity around the site of the peptide injection. It appears to be normal-looking cancellous bone, with its surfaces lined by active osteoblasts and osteoclasts. The newly formed cancellous bone is easily distinguished from the previously existing cortical bone surrounding the injection site.

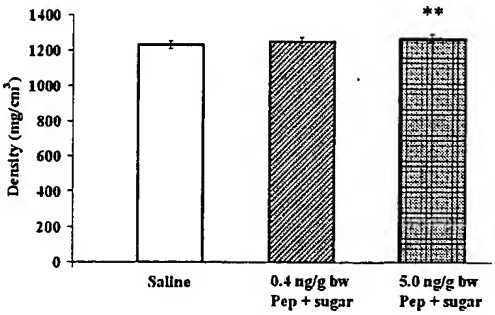

FIGURE 5. Cortical/subcortical bone density (adult study). The cortical/subcortical bone density of the midshaft of the tibia (slice 1) was significantly increased in animals treated with two doses of the glycosylated form of the 14 a.a. peptide fragment, as compared to saline-treated controls. Bars represent means (n = 8) ± SD. * = $p < 0.05$, ** = $p < 0.01$.

III. DISCUSSION

In the studies presented, Vitamin D-binding protein-macrophage activating factor (DBP-MAF) and the peptide fragments of the native serum protein appear to demonstrate an anabolic effect on the bones of the treated rats. The systemic administration of these agents elicits an osteogenic response in the bones examined, but did not show any signs of bone formation or calcifications at the injection sites. The peritoneal cavity was examined for evidence of calcification at the termination of the neonatal injection studies, and samples of tissue from the subcutaneous injection sites from the adult studies were subjected to pQCT analysis and found to contain no areas of bone formation or calcification. Although detailed safety and toxicology studies were not conducted, the treated animals showed no deleterious effects from the treatment; growth appeared normal, and gross inspection of the organs revealed no pathology.

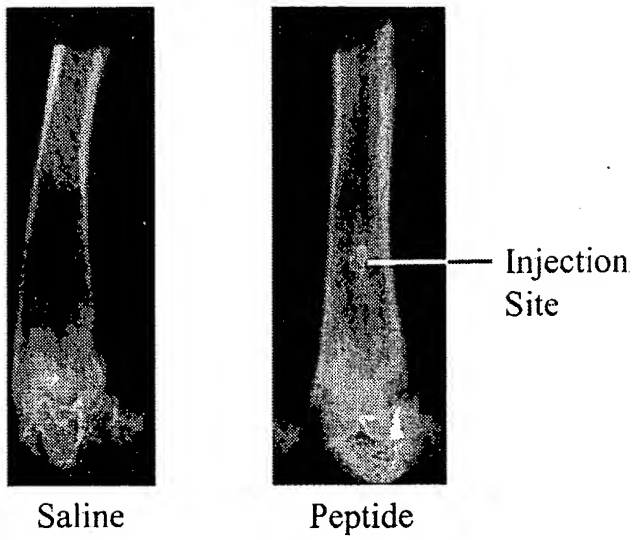

FIGURE 7. X-rays of local injection. Radiographs of the distal femur of adult rats injected with a single dose of peptide (right) or saline (left). Radiopacity in the proximity of the site of peptide injection indicates osteoinduction in the bone marrow cavity. Bone density is also increased in the areas surrounding the peptide injection in both cortical and metaphyseal bone, indicative of osteogenesis. These features are absent in the saline-injected femur.

The most dramatic effect of both the whole protein and peptide fragments was in the neonatal animals. This was to be expected, because these are rapidly growing animals with high levels of metabolic activity in their long bones. The major anabolic effects were demonstrated as an increase in total bone density and in the thickness of the shafts of the long bones. None of the systemic injection studies led to increases in length of the long bones, suggesting that the compounds are acting primarily on cells of the osteoblastic lineage and are not effecting cells of the chondrocyte lineages—for example, the chondrocytes in the growth plates. The adult animals responded to the peptide treatment in a similar fashion to the neonates, but the changes in bone density were not as dramatic. The adult animals also did not show an increase in bone diameter, as was seen in the neonates.

The peptides used in these studies were designed based on the assumption that the modified region of the native protein was the site responsible for the noted effects on the skeletal system. The

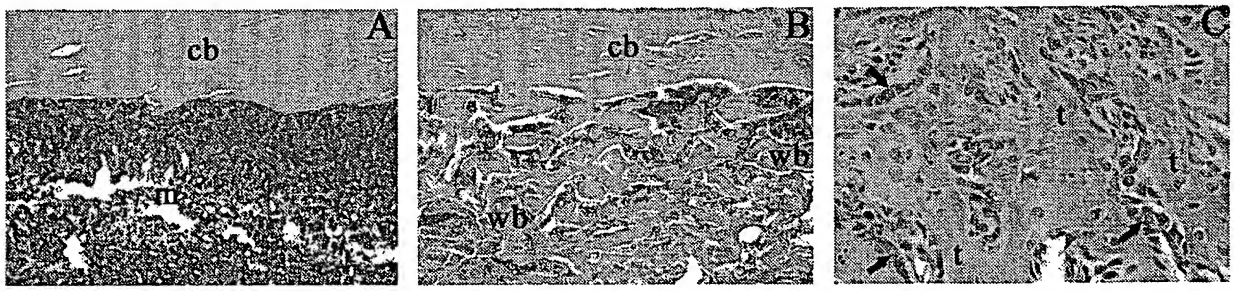

FIGURE 8. H&E stained sections of the diaphysis from control (**A**) and peptide (**B** and **C**) injected femurs. (**A**) Low-power photomicrograph showing normal cortical bone (cb) and marrow (m) in a saline/BSA-injected control femur. There was no evidence of *de novo* bone formation in the marrow cavity. (**B**) Low-power photomicrograph of a similar region shown in (**A**), but in a peptide-injected femur showing extensive formation of new, woven bone (wb) within the marrow cavity around the site of injection. (**C**) High-power photomicrograph of bone formed in response to the peptide showing bony trabeculae (t) of the newly formed woven bone lined with rows of active osteoblasts (arrows). Magnifications: **A** and **B** = 60×; **C** = 340×.

fact that the 14 amino acid peptide worked as well as the whole protein in the neonatal systemic studies provided evidence that the assumption was correct. Apparently, the modification of the native protein, by removing the terminal sugars from the glycosylation site in the third domain, uncovered a structural conformation that is responsible for the action of the DBP-MAF on the skeleton. The modified protein still contained one linked sugar residue. Our early studies used a glycopeptide, based on the assumption that the sugar was necessary for biological activity. This was not the case; the non-glycosylated peptide actually outperformed the glycopeptide in the adult systemic studies. The amino acid chain alone appears to represent the conformational backbone necessary for the peptide's influence on the skeletal system.

The systemic studies suggest that the response elicited by these agents is osteogenic, that is, bone is forming where bone had previously existed. The local injection studies provide further evidence of the effects of the peptides. New cancellous bone formed around the site of the local injection, which was directly into the red marrow of the femur. Because there was no existing bone in this region, this study suggests that the agents are not only osteogenic but may be osteoinductive as well. This appears to be selective osteoinduction, because bone was not formed at the intrapertioneal or subcutaneous injection sites. Most likely, bone marrow stromal cells in the red marrow that have the potential to differentiate into osteoblasts may be responding to the peptide signal and differentiate into mature osteoblasts and begin to lay down new cancellous bone. Unlike the bone morphogenetic proteins, which have the capacity to form bone by recapitulating the endochondral ossification cascade, these peptide signals initiate the intramembranous pathway of bone formation. New woven bone is evident by 7 days postinjection, with no cartilage present as an intermediate. The rapid induction of bone via local delivery would make the peptides potential therapeutic agents for a number of skeletal disorders.

The local administration of these agents could be used therapeutically for fracture repair (in the case of potential nonunions), the correction of bony defects, spinal surgery, and as biological additives to the devices used in joint replacement. The outcomes of the local injection studies described here point out some of the potential advantages of these peptides over the currently utilized bone morphogenetic proteins (BMPs). Although the BMPs have been proposed as potential therapeutic agents for a number of years, their clinical application and utilization has finally been realized with respect to lumbar spine fusion (Boden et al., 2002; Walker and Wright, 2002).

The results of the systemic studies suggest therapeutic opportunities for the administration of these novel anabolic peptides to treat disorders such as osteoporosis, osteogenesis imperfecta, osteopenias associated with cancer, renal dialysis, long-term glucocorticoid therapy or even space travel. To date, the most widely studied and clinically approved anabolic bone agent is parathyroid hormone (PTH) and recombinant peptide fragments of the hormone. When PTH is administered intermittently at relatively low doses, it results in an osteogenic response (Tam et al., 1982; Hock and Gera, 1992). PTH has been shown to be effective in the treatment of both men and women with osteoporosis (Kurland et al., 2000; Dempster et al., 2001). In a large study of postmenopausal women with osteoporosis, Neer and associates showed that daily subcutaneous injections of recombinant human PTH (rh PTH 1–34) resulted in a dose-dependent increase in lumbar spine bone mineral density (BMD), as well as hip and total body BMD (Neer et al., 2001). Like PTH, the peptide fragments of DBP have to be administered intermittently to elicit an anabolic effect. PTH is most effective if given on a 24-hour cycle. The DBP peptides are most effective in eliciting their anabolic effect if given on a 48-hour cycle. In fact, the DBP peptides demonstrate very little effect if administered daily.

Although recombinant human PTH (1–34) peptide is the only anabolic agent approved for clinical use, it has some disadvantages from a patient compliance perspective. The PTH drug has to be delivered as a daily injectable. It has not proven to be effective if given orally. A potential advantage of the DBP peptides is their small size. The studies described here were conducted with a 14-amino acid fragment. Preliminary studies have demonstrated that smaller peptide fragments have the same anabolic effects on bone in adult, intact rats. These peptides not only increase total bone density, but also enhance the strength of the bone. We are currently testing some of the smaller peptide frag-

ments of DBP via oral delivery to determine if these agents can be administered in pill form, thereby reducing costs and increasing compliance.

At present, the mechanism by which these novel DBP peptides initiate their anabolic effects are unknown. The effects of these potential drugs are similar to those seen with PTH therapy. We are currently evaluating the effects of the DBP peptides *in vitro*. Primary rat osteoblast and bone marrow stem cell cultures have been treated with the peptides. Proliferation and markers for osteoblastic differentiation and synthetic activity will be evaluated.

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REFERENCES

Boden S, Kang J, Sandhog H, Heller J (2002): Use of recombinant human bone morphogenetic protein-2 to achieve posterolateral lumbar spine fusion in humans. Spine 27:2662–2673.

Dempster D, Cosman F, Kurland E, Zhou H, Nieves J, Woelfert L, Shane E, Plavetic K, Muller R, Bilezikian J, Lindsay R (2001): Effects of daily treatment with parathyroid hormone on bone microarchitecture and turnover in patients with osteoporosis: A paired biopsy study. J Bone Miner Res 16:1846–1853.

Haddad J, Walgate J (1976): 25-Hydroxyvitamin D transport in human plasma: Isolation and partial characterization of calcifidiol-binding protein. J Biol Chem 251:4426–4432.

Haddad J (1982): Human serum binding protein for vitamin D and its metabolites: Evidence that actin is the DBP binding component in human skeletal muscle. Arch Biochem Biophys 213:538–544.

Hock J, Gera I (1992): Effects of continuous and intermittent administration and inhibition of resorption on the anabolic response of bone to parathyroid hormone. J Bone Miner Res 7:65–72.

Kurland E, Cosman F, McMahon D, Rosen C, Lindsay R, Bilezikian J (2000): Therapy of idiopathic osteoporosis in men with parathyroid hormone: Effects on bone mineral density and bone markers. J Clin Endocrinol Metab 85:3069–3076.

Li M, Ma Y, Jee W, Underwood R, Sietsema W (1995): An *in vivo* model for the rapid assessment of skeletal effects of anabolic agents. Bone 17:243S–247S.

Neer R, Arnaud C, Zanchetta R, Prince R, Gaich G, Reginster J-Y, Hodsman A, Eriksen E, Shalom S, Genant H, Wang Q, Mitlak B (2001): Effect of parathyroid hormone (I-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. N Eng J Med 344:1434–1441.

Popoff S, Schneider G (1994): Conversion of the vitamin D-binding protein to a macrophage activating factor: Associated defects in osteopetrotic mutations. In Norman AW (ed): Vitamin D, a pluripotent steroid hormone: Structural studies, molecular endocrinology and clinical applications. Berlin, New York: Walter deGruyter, 693–701.

Popoff S, Schneider G (1996): Animal models of osteopetrosis: The impact of recent molecular developments on novel strategies for therapeutic intervention. Mol Med Today 2:349–358.

Safadi F, Xu J, Smock S, Kanaan R, Selim A, Odgren P, Marks S, Jr, Owen T, Popff S (2003): Expression of connective tissue growth factor in bone: Its role in osteoblast proliferation and differentiation *in vitro* and bone formation *in vivo*. J Cell Physiol 196:51–62.

Schneider G, Benis K, Flay N, Ireland R, Popoff S (1995): Effects of vitamin D-binding protein-macrophage activating factor (DBP-MAF) infusion on bone resorption in two osteopetrotic mutations. Bone 16:657–662.

Schneider G, Key L Jr, Popoff S (1998): Osteopetrosis: Therapeutic strategies. The Endocrinologist 8:409–417.

Swamy N, Ghosh S, Schneider G, Ray R (2001): Baculovirus-expressed vitamin D-binding protein-macrophage activating factor (DBP-MAF) activates osteoclasts and binding of 25-hydroxyvitamin D_3 does not influence this activity. J Cell Biochem 81:535–546.

Tam C, Heersche J, Murray T, Parsons J (1982): Parathyroid hormone stimulates the bone apposition rate independently of its resorptive action: Differential effects of intermittent and continuous administration. Endocrinology 110:506–512.

Van Baelen H, Bouillon R, DeMoor P (1980): Vitamin D-binding protein (Gc-globulin) binds actin. J Biol Chem 255:2270–2272.

Viau M, Constans H, Debray H, Montreuil J (1983): Isolation and characterization of the o-glycan chain of the human vitamin D-binding protein. Biochem Biophys Res Comm 117:324–331.

Walker D, Wright N (2002): Bone morphogenetic proteins and spinal fusion. Neurosurg Focus 13:6.

Yamamoto N, Homma S (1991): Vitamin D_3 binding protein (group-specific component, Gc) is a precursor for the macrophage activating signal factor from lysophosphatidylcholine-treated lymphocytes. Proc Natl Acad Sci U S A 88:8539–8545.

Yamamoto N, Kumashiro R (1993): Conversion of vitamin D_3 binding protein (group-specific component) to a macrophage activating factor by the stepwise action of β-galactosidase of B cells and sialidase of T cells. J Immunol 151:2794–2802.

Yamamoto N, Lindsay D, Naraparaju V, Ireland R, Popoff S (1994): A defect in the inflammation-primed macrophage-activation cascade in osteopetrotic rats. J Immunol 152:5100–5107.

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